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Impact of Chronic Oral Administration of Silver Nanoparticles on Cognitive Abilities of Mice

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Abstract—To assess the effect of silver nanoparticles on mice cognitive abilities, daily, up to 4-month period, experimental mice were administrated with silver nanoparticles solution. Accumulation of silver in brain was assessed by neutron activation analysis. Cognitive abilities in mice before and after silver nanoparticles administration were evaluated in the Morris water maze behavioral test. No significant differences in the amounts of silver accumulated in brain were found between capable and incapable animals. Silver accumulation in brain of experimental animals in 4-months experiment was higher than in 2-months experiment for both groups. In the main Morris water maze behavioral test at the control points of 2 and 4 months no statistically significant differences were found in the parameters of treks between experimental and control animals.

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INTRODUCTION

Nowadays, in biology, physiology and medicine many tasks are associated with the evaluation of cognitive dysfunction caused by different extrinsic and intrinsic factors, such as neural diseases, drug toxicities, brain traumas, etc. One of the recently studied factors is the ability of metal nanoparticles of size less than 15–20 nm to cross the blood-brain barrier [1–4]. It can possibly cause an emergence of new nozological forms of occupational diseases associated with brain malfunctions, including cognitive dysfunctions, caused by the toxicity of nanoparticles to brain cells [5–8]—especially when workers in some sectors of the modern industry can be continuously exposed to nanoparticles. In occupational pathology, various methodologies to assess the risks related to exposure to different nano-sized toxic substances (silicon dust, welding spray) have been developed. However, a systematic approach to evaluation of brain dysfunction risks due to the exposure to nanoparticles has not been yet decisively established [7]. In the first report on diseases caused by nanoparticles in industrial workers, the risk of cognitive dysfunction was not considered at all [9, 10]. At the same time, a number of behavioral studies with a single injection of nanoparticles have been published [11–14].

Considering their antimicrobial, electrical and optical properties, silver nanoparticles (AgNPs) are the most common type of engineered nanomaterials found in consumer products. Beneficial properties of AgNPs also include antibacterial, antimicrobial, antifungal and antifouling activity, deodorization, non-allergenic, stain resistance, electrical conductivity, self-cleaning and antiseptic [15]. According to Janaković and co-authors [16] in 2015 AgNPs production volume ranged from 150 to 450 Mg. In 2020 the volume of produced AgNPs according to different literature sources varies from 250 to 600 tons and it will increase continuously over next several years [15].

In the present study the effect of AgNPs on cognitive abilities of mice under prolonged oral exposure was studied. In our previous study it was demonstrated that accumulation of AgNPs in various tissues without manifested visual signs of their toxic influence on the health of experimental animals [17]. The design of performed experiments imitated human occupational exposure to AgNPs in real conditions, by prolonged, systemic administration of small doses of nanoparticles with daily food intake. The amount of silver accumulated in the brain tissues after certain periods of consumption of AgNPs was determined by neutron activation analysis (NAA) and compared with the results of the behavioral test in the Morris water maze (MWM).

NAA has a number of advantages over majority of other analytical methods for biological specimens among them high sensitivity, non-destructive character, simplicity of samples preparation (no chemical pre-treatment). NAA allows determination of a wide range of elements with high precision in small samples like animal organs (e.g. lymph nodes, vena cava and aorta) the amount of which is often not sufficient for the tissue dissolution [18].

We hypothesized that the mice exposed to AgNPs for a long time would demonstrate worse results of the behavioral test, compared to control animals.

MATERIALS AND METHODS

General Layout of the Experiments

Layout of the experiments is depicted in Fig. 1. The only difference between performed experiments is the duration of the period of exposure to AgNPs: 2 months in the first experiment and 4 months in the second one. Each of these two experiments consisted of several parts. Firstly, a general sample of animals was initially tested in MWM (selective MWM test) and consecutively divided into two groups of capable and incapable animals (see in Subsection 2.5). Secondly, experimental mice were exposed to AgNPs, and control mice—to clean water, during the same period of exposure (2 or 4 months). At the end of aforementioned periods capable animals were tested in the MWM again (main MWM test). Then all animals were euthanized, and brain and blood samples were extracted for the estimation of silver content in them by NAA. Eleven experimental mice (8 capable and 3 incapable) and 15 control mice (10 capable, 5 incapable) after 4-months experiment were used for the estimation of AgNPs' excretion. After the main MWM test at the end of the 4-months period (and for incapable animals—at the same point) they consumed clean water during additional 2 months. Then capable mice were tested in MWM for the third time, and euthanized with the extraction of brain and blood samples for NAA.

Animals and Dosage

The experimental AgNPs' solution was prepared from the purchased concentrated (13 mg/ml) colloid solution of AgNPs ("Argovit-C", SPC *Vector-Vita*, Russia) containing nanoparticles of 34 ± 1.4 nm in a diameter (mean $\pm$ SD) dispersed in distilled water and coated with a stabilizing coating of polyvinylpyrrolidone. Particle size distribution of the Argovit-C colloidal preparation as well as TEM images of nanoparticles can be found in [8, 19]. Size of the AgNPs was confirmed by photon correlation spectroscopy in the National Research Centre "Kurchatov Institute". For experiments initial solution was diluted with water at a ratio of 1 : 500.

Outbred white mice (SHK) aged from three to four months (with a mean $\pm$ SD bodyweight of 30.03 ± 6.42 g, 34.71 ± 5.13 g in males and 27.51 ± 5.64 g in females) were used in experiments. Mice are heterozygous by undefined number of genes and are used to assess the safety of medical and cosmetic products, and dietary supplements. The first experiment included 47 male and 69 female mice, the second one—87 and 89, respectively. Animals were supplied by two farms in the Moscow region (Russia), the *Andreevka Farm* for the first experiment and the *Stolbovaya Farm* for the second one. The animals were housed in the vivarium of Moscow Regional Research and Clinical Institute named after M.F. Vladimirsky. Mice were kept in steel cages ($31.5 \times 23 \times 15.7$ cm) in groups of 3 to 4 mice in each cage, with natural light cycle and ambient temperature of $22\text{--}24^\circ\text{C}$, and with *ab libitum* access to food and liquid (experimental SN solution or water). Each cage was cleaned once a day. The methodology of the experiments and the maintenance of the animals in the institutional vivarium had been approved by local Ethics Committee and met the requirements of the Directive 2010/63/EU of the European Parliament and of the Council from September 22, 2010 on protection of animals used for scientific purposes and in compliance with the American College of Toxicology Policy on the Use of Animals in Toxicology.

In this study we modeled continuous oral administration of small daily doses of the potential toxic agent. During the first experiment, AgNPs were administered for 2 months, and during the second for 4 months. The experimental mice were drinking the solution with concentration of AgNPs $25 \mu\text{g}$ per 1 mL of water, so the total daily intake was $100 \mu\text{g}$ per animal (the daily norm of liquid intake per mouse is 4 mL [20]). Control mice were drinking water. During the experiments 4 animals (3 males, 1 female) were eliminated because of natural causes. All the animals were visually healthy and behaved normally.

Preparation of Samples and Weighting

At the end of the period of AgNPs administration the incapable mice and a portion of the capable mice (see below) were euthanized, whereas the majority of the capable animals proceeded into the second test, and after that they were euthanized, as well. To estimate the excretion of silver, a portion of the experimental mice were drinking water during two months after the second test in the second experiment. Animals that were used for estimation of excretion of silver were euthanized after the third MWM test. Total body mass (TBM) just before the euthanasia was measured.

The euthanasia was performed by an intraperitoneal injection of the urethane solution in water at a dose concentration of 1.2 g of dry urethane per kg of the body weight, prescribed by a veterinarian. Brain (whole) and blood samples were taken from each

Fig. 1. Layout of the experiments.

mouse; individual brain mass (BRM) of a freshly cut brain was measured in grams, and the 1–1.5 mL of blood were collected. Individual percentages of brain mass to body mass ($100\% \cdot \text{BRM}/\text{TBM}$) were calculated. After measurement of weight, tissue samples were packed in aluminum foil, dried for 24 h at a temperature of 75°C.

The percentages of the brain to body mass ($100\% \cdot \text{BRM}/\text{TBM}$) were compared for the capable and incapable mice (see below). Influence of the factors “group” and “gender” on these percentages was assessed by multivariate analysis of variance (two-tailed MANOVA) with a post-hoc analysis by Fisher LSD test, after the normality of the distribution has been confirmed by Shapiro-Wilk test. For each gender and in each group, mean values, SD, 95% confidence intervals, medians and quartiles were calculated.

Measurement of the AgNPs Accumulation in the Brain

Prepared samples were transported to the Frank Laboratory of Neutron Physics (FLNP) of the Joint Institute for Nuclear Research (JINR) in Dubna (Russia) to determine silver content in every sample by neutron activation analysis (NAA). This is a sensitive and non-destructive technique useful for quantitative multi-elementary analysis of the major, minor, and trace elements present in various matrices [21, 22].

During the preparations for NAA the dried samples were weighed again (dry weigh) and repacked in aluminum foil for epithermal neutron activation analysis (ENAA) at the REGATA installation of the pulsed fast reactor IBR-2. The description of irradiation channels and pneumatic transport system of the REGATA unit at IBR-2 has been given in the paper by Pavlov et al. [22]. The temperature in the irradiation channels of

the IBR-2 reactor does not exceed 60–70°C that allows for the analysis of biological samples without their damage. Seventy-nine (79) blood samples and 79 brain tissue samples were irradiated sequentially in batches for 24 and 48 h, respectively, each batch consisting of six aluminum containers simultaneously. Two standard reference materials (SRM) 2710 (Trace elements in soil (NIST, USA) and 2891 (Cupric Sandstones, polymetallic ores, Karaganda, Kazakhstan (LLP “Centrgeolanalit”)) were irradiated within each transport container with the studied samples. The uncertainty level for the silver determination in SRM 2891 was 10%. To determine the concentration of silver, the $\text{Ag}^{110\text{m}}$ isotope was used. Neutron flux density of the thermal neutrons was $1.6 \times 10^{12} \text{ n cm}^{-2} \text{ s}^{-1}$. Gamma spectra of induced activity were measured with three spectrometers based on HPGe detectors with an efficiency of 40–55% and a resolution of 1.8–2.0 keV for ^{60}Co and Canberra spectrometric electronics. All spectrometers were equipped with automatic sampling changers [22].

All irradiated samples and SRMs were measured in the same geometry. Gamma spectra were measured several times at 7 and 20 days after irradiation for 1.5 h. Some of the samples with small statistics for silver gamma lines were additionally measured for 5 h. Analysis of the spectra and calculation of isotope activity was carried out with the use of Genie2000 software (Canberra Industries (Moscow, Russia)) with the verification of the peak fit in an interactive mode. The concentration was calculated using the $\text{Ag}^{110\text{m}}$ isotope with a half-life of 249.8 days in a batch mode, with software developed at Joint Institute for Nuclear Research [22]. The calculation was carried out for the $^{110\text{m}}\text{Ag}$ 657.8 (94.6%), 763.9 (22.2%), and 937.5 (34.3%) keV peaks (the probability of gamma quanta yield is given in parentheses). In this case, a correction was made for possible interference with the isotope ^{76}As ($T_{1/2} = 1.1$ days, 657.1 keV). Quality control of analytical results was provided by comparing the calculated and recommended (passport) concentrations for the standard reference materials.

Potential influence of the factors “duration of AgNPs administration” (2 or 4 months), “gender” and “group” (belonging to groups of individuals with different levels of cognitive abilities, see below) on the content of silver in brain of the experimental mice was estimated by multivariate analysis of variance (two-tailed MANOVA) with post-hoc analysis by Fisher LSD test. The normality of distribution has been confirmed by Shapiro–Wilk test. For each group and subgroup both mean and standard deviation (SD) were calculated.

Assessment of Cognitive Abilities

Morris water maze (MWM) test was used to assess cognitive abilities in mice [23]. The circular white pool

(1.5 m in diameter) was filled with water, and various visible clues were positioned around the pool. Water was made opaque with dry milk to hide the underwater platform. Each animal had to find this invisible underwater platform on which it could escape from water. Location of the platform remained constant all time. If the animal did not find the platform during a trial, it was gently placed on the platform manually. Each test was conducted with small groups of 12–15 animals and consisted of three consecutive days with three trials daily. During each trial, a mouse was allowed to freely swim in the pool for a maximum of 180 s. When the animal either found the platform or was placed on it after the time was up, it was left to sit on the platform for 20 s, and then it was picked up, dried with a clean cloth and returned to its cage until its next trial. Therefore, each animal had 20–30 min of rest between the trials.

Video records of each test were analyzed by Ethovision program (Noldus Information Technology, Netherlands). Trajectories (tracks) of all trials were recorded and visualized (Fig. 2). Based on those, three parameters were calculated for each trial: the distance moved (track length (cm), the latency to platform (sec) and the total distance to platform (cm)). The last parameter is a sum of distances to platform from all points of the track: if the value is low, this means that a mouse was swimming near the platform. Means of the parameters per a day were calculated for each animal, with subsequent comparison of the daily samples of the means.

For each animal in each experiment we determined the behavioral type that was demonstrated during trials in both the selective and the second tests. Based on the results of the selective test, the general sample was divided into two groups: “incapable” and “capable” animals (Fig. 1). Incapable mice demonstrated types of behavior, which did not include an exploration of the pool, i.e. thigmotaxis, or swimming along the walls of the pool, and drift, or laying on the surface without any movement. Therefore, these mice rarely found the platform due to the lack of exploratory activity; so, this group was excluded from the tests as being non-responsive to potential changes induced by SN. All other individuals (71 in the 2-month experiment and 102 in the 4-month experiment) demonstrated various types of behavior that included an exploration of the pool (random search, directed search, “scanning”), so they were assigned to the group of capable animals (capable *sensu lato*, i.e., both capable and intermediate groups from the previous study [24]) and followed into the second test. Each group included animals of both genders.

In each experiment, the group of capable animals was randomly divided into two subgroups, the experimental and control one, both containing males and females (Fig. 1). In total, 33 mice (17 experimental and 16 control) participated in the main test of the

Fig. 2. Different types of behavior in the Morris water maze test—examples of the trajectories: (1–3) directed search; (4–6) random search; (7–9) thymotaxis.

experiment with two months of AgNPs administration, and 62 mice (35 experimental and 27 control) participated in the second test of the second experiment, with four months of AgNPs administration. Twenty-six (26) mice (11 experimental and 15 control) participated in the third test in the study of excretion of silver.

The results were analyzed using Statistica 13.2 software (Dell inc., USA), at the significance level of $p < 0.05$; all tests were two-tailed. Distribution of quantitative variables was checked for normality by Shapiro-Wilk test; this hypothesis was rejected for all three parameters. In each test the daily changes of all three parameters (learning curves) for each subgroup were assessed by Wilcoxon matched pairs test at days 1 and 3. Between subgroups differences for three parameters were assessed daily by Mann–Whitney U -test. The Δ (percentage of the difference between medians at Day 3 and Day 1 to the median at Day 1) indicates the inclination of the learning curve: it was calculated for

each parameter in each subgroup and compared between two tests. Results obtained from male and female mice were compared in each subgroup by Mann–Whitney U -test.

RESULTS

Accumulation and Excretion of Silver

The amounts of silver accumulated in the brain (i.e. AgNPs which crossed the blood-brain barrier) after various periods of the AgNPs administration are shown in Tables 1 and 2. As it can be seen from the data presented in Table 1, excretion of the silver from the brain was slow. These data are in agreement with the results of our previous studies [17]. It should be noted that in both experiments the relatively small amounts of silver were detected in the brain samples of the control animals (Table 1). Similar findings were observed in several studies with the oral AgNPs intake [25, 26].

Table 1. Amounts of silver accumulated in the brain and blood samples determined by NAA, in ng/g (mean $\pm$ SD)

Subgroups of animals		Duration of administration		
		2 months (20 experimental and 10 control mice)	4 months (24 experimental and 4 control mice)	2 months of excretion after 4 months of nanoparticle administration (11 experimental mice)
Brain				
Experimental	Mean	319 ± 155	870 ± 200	837 ± 227
Control	Mean	45 ± 35	185 ± 13	—
Blood				
Experimental	Mean	2.25 ± 1.9	1.44 ± 0.5	0.45 ± 0.4
Control	Mean	0.32 ± 0.21	0.09 ± 0.06	—

Table 2. Amounts of silver accumulated in the brain samples determined by NAA, in ng/g (for groups assessed in the MWM by the cognitive abilities, the experimental mice only) (mean $\pm$ SD)

Groups of animals		Duration of administration		
		2 months (3 capable and 4 incapable mice)	4 months (6 capable and 10 incapable mice)	2 months of excretion after 4 months of the intake (5 capable and 3 incapable mice)
Capable	Mean	401 $\pm$ 114	832 $\pm$ 229	849 $\pm$ 193
Incapable	Mean	387 $\pm$ 215	929 $\pm$ 128	968 $\pm$ 309

The duration of AgNPs administration significantly influenced the differences between the samples from the experimental animals of two-month and four-month experiments ($p < 0.00001$; $F = 102.7$; $df = 1$). Neither gender nor group as factors had any significant influence ($p = 0.085$ and $p = 0.817$ respectively; $F = 3.1$ and $F = 0.05$ respectively; for both $df = 1$). The interaction (cross-effect) of gender and duration of the AgNPs administration as factors was not significant ($p = 0.188$; $F = 1.8$; $df = 1$). However, the 95% confidence intervals for the gender samples in the 2-month experiment overlapped twice as much, as those in the 4-month experiment (Fig. 3). At the pairwise comparison, significant differences in the accumulated amount of silver in the brain were also observed between these two experiments. The amounts of silver accumulated in the brain of male and female mice in the 2-months experiment differed significantly, but

the gender was unlikely the cause of this difference (Table 3).

Cross-effect between the group and the duration of AgNPs administration as factors was insignificant ($p = 0.068$; $F = 3.5$; $df = 1$). However, means and confidence intervals in the 2-months experiment were higher for the capable mice; while in the 4-months experiment, they were higher for the incapable animals (Fig. 4; Table 2). At the pairwise comparison, significant differences were found between two experiments, but not between the experimental and control groups within each experiment (Table 4). In capable mice there was no correlation between parameters measured in MWM and the silver content in the brain. Also, it should be noted that the behavior and physical appearance of experimental animals were identical with those of the control mice, thus indicating no significant impact of the chosen daily dose of silver on animals' health.

Table 3. Statistical significance of the gender differences in specific mass contents of silver in the brain (post-hoc Fisher LSD test)

Duration of nanoparticle administration	Gender	2 months		4 months	
		males	females	males	females
2 months	Males	X	0.0453	$<10^{-4}$	$<10^{-4}$
	Females	0.0453	X	$<10^{-4}$	$<10^{-4}$
4 months	Males	$<10^{-4}$	$<10^{-4}$	X	0.7496
	Females	$<10^{-4}$	$<10^{-4}$	0.7496	X

Fig. 3. Specific mass content of silver in brain after different periods of administration of AgNPs in males (square points) and females (circle points). Legend: point values show the group mean; interval, 95% confidence interval.

Fig. 4. Specific mass content of silver in brain after different periods of administration of AgNPs in the capable (square points) and the incapable (circle points) mice. Legend: point values show the group means; interval, 95% confidence interval.

Fig. 5. Learning curves obtained in the experimental mice in the 2-months experiment: (a) selective testing (before administration of AgNPs), (b) the second testing (after administration of AgNPs). The central point represents the median; boxes, the lower and upper quartiles; intervals, the ranges.

MWM Results in the Selective Test

In the selective test of each experiment, the learning curve typical for MWM was obtained for the group of capable animals and for its both subgroups: daily medians and means of all three parameters were declining (Figs. 5–8, a). In each experiment, parameters' values from Day 1 and Day 3 of the selective test were compared, and in each group of capable animals the differences were statistically significant for all three parameters: the distance moved ($p = 0.000001$ in the 2-months experiment and $p < 0.00001$ in the 4-months experiment), the latency to the platform ($p = 0.023$ and $p < 0.00001$ respectively) and the total distance to the platform ($p = 0.000018$ and $p < 0.00001$ respectively).

At similar comparison done separately for each subgroup, the differences for the distance moved and the total distance to the platform were statistically significant in both the experimental (in the 2-months experiment $p = 0.0002$ and $p = 0.0004$ respectively; in the 4-months experiment both $p < 0.00034$) and the control (in the 2-months experiment $p = 0.0018$ and $p = 0.0215$ respectively; in the 4-months experiment both $p < 0.0014$) subgroups, with the exception of the latency to platform. For this parameter in the 2-months experiment the differences were insignificant ($p = 0.112$ in the experimental subgroup and $p = 0.095$ in the control subgroup), but in the 4-months experiment they were significant ($p < 0.00034$ in the experimental subgroup and $p < 0.0014$ in the control subgroup). Also, all experiment parameters' values

Table 4. Statistical significance of the differences in specific mass contents of silver in the brain between the groups by cognitive abilities (the experimental mice only): post-hoc Fisher LSD test

Period of admission of nanoparticles	Group of animals by cognitive abilities	2 months		4 months	
		Incapable	Capable	Incapable	Capable
2 months	Incapable	X	0.1724	$<10^{-4}$	$<10^4$
	Capable	0.1724	X	$<10^{-4}$	$<10^{-4}$
4 months	Incapable	$<10^{-4}$	$<10^{-4}$	X	0.2123
	Capable	$<10^{-4}$	$<10^{-4}$	0.2123	X

Fig. 6. Learning curves obtained for the control mice in the 2-months experiment: (a) selective testing (before administration of AgNPs), (b) the second testing (after administration of AgNPs). The central point represents the median; boxes, the lower and upper quartiles; intervals, the ranges.

Fig. 7. Learning curves obtained for the experimental mice in the 4-months experiment: (a) the selective testing (before administration of AgNPs), (b) the second testing (after administration of AgNPs). The central point represents the median; boxes, the lower and upper quartiles; intervals, the ranges.

Fig. 8. Learning curves obtained for the control mice in the 4-months experiment: (a) the selective testing (before administration of AgNPs), (b) second testing (after administration of AgNPs). The central point represents the median; boxes, the lower and upper quartiles; intervals, the ranges.

from each day were compared between two subgroups: no differences were found for them (all $p > 0.1$).

MWM Results in the Main Test

In the main test experiment parameters' values from each day were compared between the experimental and control subgroups, and no statistically significant differences were found for all parameters (all $p > 0.3$) (Figs. 5–8, b). It should also be noted that in each experiment the daily medians and means of the parameters measured at Day 1 of the main test were close to those measured at Day 3 of the selective test (Figs. 5–8).

However, in each experiment the day-by-day dynamics of the parameters in the main test differed between two subgroups. At the same time, it changed in each subgroup in comparison with the results of the selective test. In the experimental subgroup of 2-months experiment the distance moved, as well as the total distance to the platform, were declining in the main test more smoothly than in the selective test (at Day 3, the respective Δ were -41.2 and -63.2% in the selective test and -24.9 and -33.6% in the second test). But in the control subgroup of 2-months experiment these two parameters declined in the selective test, but slightly increased in the main test (at Day 3, the respective Δ were -31.1 and -21.2% in the selective test and 4.7 and 8.7% in the second test). In com-

parison to the results of the selective test the latency to platform in the main test increased in the experimental subgroup (at Day 3, the Δ were -24.7% in the selective test and 85.8% in the second test) and was decreasing more smoothly in the control subgroup (at Day 3, Δ were -16.1% in the selective test and -9.3% in the second test).

In 4-months experiment the differences in day-by-day dynamics of the parameters between the main test and the selective test in the experimental subgroup were slight: the decrease in the distance moved and in the latency to platform remained roughly the same (at Day 3, the respective Δ were -37.1 and -34.4% in the selective test and -39.4 and -33.4% in the second test), whereas the total distance to the platform declined more smoothly in the main test (at Day 3, the respective Δ were -48.9% in the selective test and -31.7% in the second test). However, in the control subgroup of the 4-months experiment, three parameters declined in the selective test but increased in the main test (at Day 3, the respective Δ were -21.7 , -25.4 , and -34% in the selective test and 26.7 , 48.2 , and 48.9% in the second test).

MWM Results after the Excretion of the Silver

After two months of drinking clean water by experimental mice, statistically significant differences between the experimental and control animals were

Fig. 9. Learning curves obtained in the third testing after 2 months of excretion of silver: (a) the experimental mice, (b) the control mice. The central point represents the median; boxes, lower and higher quartiles; intervals, the ranges.

found only at Day 1 of the test after excretion (the distance moved, $p = 0.036$; the latency to platform, $p = 0.027$; the total distance to the platform, $p = 0.021$). The daily values of these parameters did not show any significant changes in any of the subgroups (Fig. 9). It should be noted that in comparison to the results of the main test of the 4-months experiment, the daily values of the parameters in the test after excretion tended to be lower and to have smaller ranges, while the values at Day 1 of the test after excretion were similar to those at Day 3 of the main test (Figs. 7–9).

Gender Differences in the MWM Results

In the selective test of the 2-months experiment, there were no gender differences for all parameters in the general sample (all $p > 0.136$, with the exception of a tendency in the latency to platform at Day 3, $p = 0.05$) and in both subgroups (all $p > 0.136$, with the exception of the distance moved at Day 2, $p = 0.041$, and a tendency in the latency to platform at Day 3, $p = 0.058$, in experimental subgroup). And in the main test gender differences were absent for all parameters in both subgroups (all $p > 0.171$). In the selective test of the 4-months experiment gender differences were not found in all parameters in the general sample (all $p > 0.097$) and in both subgroups (all $p > 0.09$). At the same time, in the main test gender differences in the experimental subgroup were statistically significant only in the distance moved at Day 2 ($p = 0.0234$), but

in the control subgroup at Day 3 gender differences in two of three parameters were significant (the distance moved at Day 3, $p = 0.045$, the latency to platform at Day 3, $p = 0.027$), with lower values in males.

Brain Mass, Body Mass and Cognitive Abilities

As a collateral result, we found that more capable mice of both genders had a significantly larger brain mass relatively to the total mass of the body: these percentages increased from those in the incapable mice to those in the capable mice in each gender sample and in the general sample, as well (Table 5). Both gender and the group as factors had a significant influence on the differences between the samples ($p = 0.00031$ and $p = 0.0085$ respectively; $F = 16.7$ and $F = 7.9$ respectively; both $df = 1$). However, the cross-effect between the group and gender as factors was non-significant ($p = 0.308$; $F = 1.1$; $df = 1$), and the confidence intervals were almost not overlapping in all four combinations of these two factors (Fig. 10). In pairwise comparisons, capable females were significantly different from all other combinations; no significant differences between groups were found in males (Table 6).

DISCUSSION

Ingestion is an important route of AgNP exposure. It is probable that the size, shape, stability or aggregation state of the nanoparticles can change once they

Fig. 10. Percentages of brain to body mass in the capable and the incapable mice, males (squares) and females (circles). Legend: point, the group mean; interval, 95% confidence interval.

reach the stomach [27]. In vitro studies, without presence of food components, showed that AgNPs may indeed overcome the gastrointestinal juices in their particulate form without forming large quantities of aggregates, or their insignificant dissolution will take place due to limited residence time in stomach (10–240 min) [27, 28]. And in the study of the impact of digestion, with and without food additives, on colloidal stability of AgNPs, complete aggregation of AgNPs was shown in the absence of food, and only insignificant aggregation was observed in the presence of food [29]. Since in the present study experiments were performed in vivo and animals were fed *ab libitum*, it was assumed that aggregation of AgNPs in the stomach will be inconsiderable.

In order to evaluate the toxicity of AgNPs on animals, nanoparticles at concentration range from 5 to 200 $\mu\text{g/mL}$ were used in different research [30, 31]. In

our preliminary experiments solution with concentration of AgNPs 50 $\mu\text{g/mL}$ was used. However, with that concentration the mortality rate among the experimental animals during the period of AgNPs administration was very high: 65% of mice died before the second testing, and the rest of animals looked unhealthy. It was concluded that the daily intake dose of AgNPs could be too high for relatively safe (without acute reactions) chronic consumption. Thus, at the present research the concentration of the experimental solution was halved. At AgNPs concentration 25 $\mu\text{g/mL}$ no differences in mortality rates between experimental and control groups during the period of AgNPs consumption were observed (0–2%).

Published data on the long-term preservation of memory formed in the MWM test, are variable: periods of two [32] or three months [33] are mentioned.

Table 5. Percentages of the brain mass to body mass ratios by cognitive abilities

Gender	Group	Number of animals	Mean $\pm$ SD, %	95% CI	Me, %	Q1; Q3, %
Male	Capable	8	1.25 $\pm$ 0.21	1.08; 1.43	1.12	1.18; 1.37
	Incapable	5	1.08 $\pm$ 0.15	0.89; 1.26	0.95	1.13; 1.19
Female	Capable	12	1.76 $\pm$ 0.37	1.53; 1.99	1.48	1.71; 2.02
	Incapable	9	1.38 $\pm$ 0.22	1.21; 1.55	1.27	1.34; 1.57
Both genders	Capable	20	1.56 $\pm$ 0.18	1.29; 1.83	1.51	1.22; 1.79
	Incapable	14	1.27 $\pm$ 0.24	1.11; 1.43	1.25	1.12; 1.42

Table 6. Statistical significance of the differences in the percentage of brain to body mass ratios between the groups by cognitive abilities: post-hoc Fisher LSD test

		Females		Males	
		incapable	capable	incapable	capable
Females	Incapable	X	0.0037	0.0587	0.3556
	Capable	0.0037	X	0.0001	0.0003
Males	Incapable	0.0587	0.0001	X	0.2703
	Capable	0.3556	0.0003	0.2703	X

Therefore, in present experiments two- and four-month periods of the AgNPs administration were chosen.

Previously, we have proposed and used a selective test, in which we justified the necessity to divide the initial sample of animals according to identified cognitive abilities (in the sample of 52 mice) [34]. As a result, significant heterogeneity of individual characteristics of orientation and spatial memory was found. Therefore, it was necessary to identify homogeneous groups within the initial sample to prevent errors and to increase sensitivity of the method. A type of behavior exhibited in the selective test was used as a criterion for assigning an animal to a certain group: various behavioral types indicated different levels of orientation and spatial memory [36, 37]. Each behavioral type was determined by an individual ability to learn the position of the platform in the pool, and it could be confirmed by quantitative parameters of the animals' movement. Behavioral types during each trial were determined empirically by visual comparison of each trajectory with examples of the strategies taken from the paper by Garthe et al. [24] and selection of the closest version (even though characteristics of several behavioral types could be seen during a trial). The attribution of the individuals to the certain groups was based on behavioral types identified at Day 3 of the selective test.

Initially, the comparison of the data obtained for experimental and control animals had been planned only for the second test, while the selective test was performed only to divide the general sample into more homogenous groups. However, in both experiments the daily values of the three parameters in both subgroups in the second test decreased, compared to those in the selective test. This indicates a significant effect of memory, i.e. those animals, once having been tested in the MWM, preserved the information about the test facility, the structure of its inner space and positions of external clues. It was additionally proven by different (decreased or increased) inclines of learning curves and smaller ranges of the daily values of the parameters in the second test: the daily volume of mastered information was different from that in the selective test. The third test in the study of excretion also confirmed this fact: the daily means of the parameters tended to be smaller than in the second test, and

did not change much day by day, indicating that the learning had reached its limit. The presence or absence of AgNPs in the body had no clear impact on the memory effect: the differences in the dynamics and ranges of the parameters described above were found both in the experimental and the control subgroups. Nevertheless, the changes of the two space-related parameters (the distance moved and the total distance to the platform) over time in the second test were different: they were declining in the experimental subgroup, but increasing in the control subgroup.

The majority of individuals did not change their types of behavior in the second test. It indicates a strong influence of hereditary traits on the choice of the search strategies. This fact also confirms the necessity of dividing the general sample into several groups of individuals with similar levels of cognitive abilities in order to remove the risk of a wrong interpretation of the test results. It must be noted that animals changed their strategies that required a higher degree of spatial orientation to the strategies that required a lower degree of spatial orientation (i.e. directed search to random search and random search to thigmotaxis). The causes of this phenomenon remain unclear, but the presence or absence of AgNPs in the body does not seem to be one of them: such individuals were found both in experimental and control subgroups. The small difference in their numbers most likely does not exceed the limit of fluctuations among a total number of animals in the subgroups.

A few gender differences that were found in both experiments were not systematic and were probably related to the random distribution of individuals of both genders between the groups. This is indicated by the absence of any significant differences before division of the group of capable animals into the subgroups and also by the absence of differences in any parameters at all three days in both tests. In addition, the differences in two parameters at one day were found only once, and both genders mostly preserved their types of behavior. It should also be noted that in the second test these differences were either absent (in the 2-months experiment) or found both in the experimental and the control groups (in the 4-months experiment). Thus, no behavioral links between the gender and presence/absence of AgNPs in the body

were observed. So, in this case, gender as a potential factor influencing the MWM results under this protocol was weak.

Thus, in this study design, the influence of AgNPs on the results of MWM test was likely veiled by the memory effect. Both the experimental and the control animals used their previously formed (i.e. before the admission of AgNPs) experience during the second test. To avoid this effect, it was suggested that it should be necessary to change the methodical approach for further studies by excluding the preliminary selective test to prevent the formation of memory about the MWM test before the influence of any experimental factors. It could be done as follows: without a selective test, after administration of AgNPs both experimental and control animals could be tested in MWM to determine the numbers of capable vs. incapable individuals. The significant difference between these numbers in experimental and control samples should be observed, then the impact of the administration of AgNPs would be proved. It should also be noted that the selective test can be recommended for those studies which estimate influence of some given factors on the brain structure and/or development, but do not require any post-exposure (i.e. second) evaluation of cognitive abilities. In such a case, division of general sample consisting of random numbers of individuals with various levels of cognitive abilities into more homogeneous groups could significantly reduce the risk of a wrong interpretation of the results.

To control the correct distribution of animals into the groups, it is possible to use individual percentages of the brain to body mass ($100\% \cdot \text{BRM}/\text{TBM}$) as an additional morphological criterion. As an explorative result of the study, we found an association between these percentages and the level of cognitive abilities of individuals: individual percentages of the brain to body mass have been increasing in groups in parallel to the increase in degree of successful performance of the test. The latter is largely related to the behavioral strategies demonstrated in the test. Also, these percentages in males are smaller than in females, which can be caused by larger body mass in males.

Most of the studies on correlations of brain characteristics with other individual parameters have evaluated associations between the brain and the body mass and/or the body mass index (i.e. also including height). At the intraspecies level, mostly in humans, correlations between high/excessive body mass and brain volume [37], and between body mass and white matter functioning [38] have been detected. In a rat study, there were individuals with low, medium and high brain mass in each group identified by body mass [39]. There is less data on any associations of the brain mass and behavior within the species. A series of experiments has been published, which includes two independent directions for selection of mice by different qualitative features, i.e. the brain mass (high and

low) [39, 40] and the ability to perform simple logical tasks [33]. However, the relationships between the brain mass and cognitive abilities are more complex than the direct correlation [41].

As follows from the above, generally, an increased BRM index is recognized in more “clever” mammals. However, its correlation with MWM results reflecting cognitive abilities is unknown. It opens the way to develop additional quantitative and objective criterions to assess cognitive capabilities of animals, as well as to select them correctly in the MWM.

CONCLUSIONS

The study showed the continuous accumulation of silver in the brain due to a prolonged exposure. The excretion of the silver after a cessation of the exposure was also observed. No statistically significant differences in the results of the Morris water maze test between the experimental and control animals were found. In addition, no systematic gender differences both in the accumulation of AgNPs and in the behavioral test results were seen, thus, the gender as a potential factor influencing the MWM results obtained in MWM seems unlikely. However, a significant effect on memory was found: animals retained the experience acquired in the selective test and used it in the second test, both at 2 and at 4 months of administration after the selective test. Thus, the influence of AgNPs on the results of MWM test might be blunted by the memory effect. To avoid it, the changes of the methodology for future studies are proposed as follows: the selective test must be excluded to prevent the preliminary exposure to the MWM, and the individual percentages of the brain to body mass can be used as an additional morphological criterion to evaluate the correctness of animal selection into capable and incapable groups.

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DECLARATION OF INTERESTS

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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